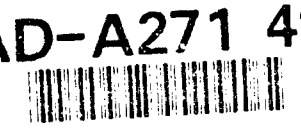

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COMPATIBILITY AND EFFICACY OF BIOCIDES QUALIFIED UNDER MILITARY SPECIFICATION MIL-S-53021

INTERIM REPORT
BFLRF No. 282

By

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The Army has three fuel biocides qualified under specification MIL-S-53021. The military's aviation kerosene, JP-8, also contains an icing inhibitor additive that has some biocidal activity. The relative effectiveness and compatibility of these additives have never been investigated. This study was conducted to evaluate the efficacy of the individual biocides as well as the possible interactions (either synergistic or antagonistic) between these biocides and the icing inhibitor additive. Each of the qualified biocides was evaluated using standard microbiological techniques to determine additive efficacy and compatibility with other fuel additives. The relative effectiveness of the qualified additives was determined. The interactions of the biocides with the icing inhibitor additive in JP-8 were also investigated.

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EXECUTIVE SUMMARY

Problems and Objectives: The Army has three fuel biocides qualified under specification MIL-S-53021. The military's aviation kerosene, JP-8, also contains an icing inhibitor additive that has some biocidal activity. The relative effectiveness and compatibility of these additives have never been investigated.

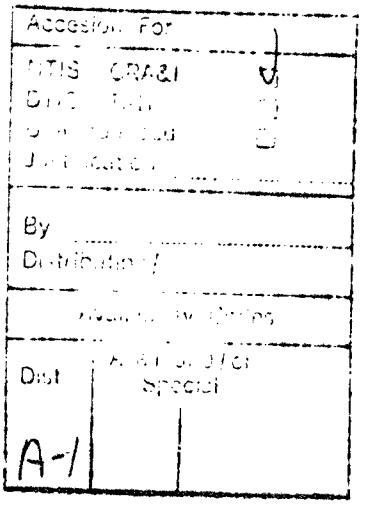
This study was conducted to evaluate the efficacy of the individual biocides as well as the possible interactions (either synergistic or antagonistic) between these biocides and the icing inhibitor additive.

Importance of Project: The most frequently reported fuel-related problem is plugged fuel filters. One of the most effective fuel filter plugging contaminants is microbiological growth. Since keeping water out of a fuel cell is, in many cases, nearly impossible, the next most effective method of controlling microbiological growth is with biocide additives. In addition to controlling microbiological growth, the additives must not adversely effect the fuel in any manner.

Technical Approach: Each of the qualified biocides was evaluated using standard microbiological techniques to determine additive efficacy and compatibility with other fuel additives.

Accomplishments: The relative effectiveness of the qualified additives was determined. The interactions of the biocides with the icing inhibitor additive in JP-8 were also investigated.

Military Impact: The results of this study should allow the end user of MIL-S-53021 biocides to specify a particular additive based on his or her needs. Since the sterilization rates of the additives differed, different additives might be specified based on the urgency of the use. The results also demonstrate that the biocides in MIL-S-53021 will not adversely react with the additives in JP-8.



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I. INTRODUCTION

Storage stability and cleanliness are requirements imposed on military fuels at the time of purchase as well as use. These requirements are necessary because of a need to ensure that fuels will be satisfactory for use regardless of the time interval between refinery production and ultimate use. This fuel quality becomes critical in relation to prepositioning of fuel stocks, either in bulk storage or in vehicle fuel cells, wherein good fuel quality must be maintained. Without guaranteed maintenance of fuel cleanliness and stability, fuels could prove to be the weak link, leading to engine malfunctions (i.e., injector sticking, nozzle fouling, filter plugging, etc.), increased fuel system maintenance problems, equipment vulnerability, decreased mobility, and numerous other problems. Although fuel-related problems such as fuel injector seizures, generator set malfunctions, and fuel system corrosion have been reported, the most frequently reported problem is fuel filter plugging.

Each of these problems may be the result of one or more factors. For example, diesel fuel-consuming vehicle and equipment fuel systems circulate the fuel as an injector coolant during operation. The fuel system also breathes air containing oxygen and water vapor and serves as a reaction vessel of undefined composition and dimension. Under these conditions, the fuel can undergo both thermal and oxidative degradation. The products of this degradation can not only plug filters but also increase corrosion of fuel-wetted surfaces. Additionally, water can collect in a vehicle fuel cell for several reasons (e.g., condensation, rain, cleaning, etc.). Fuel tank design usually prohibits complete fuel tank drainage, which means that water bottoms cannot be completely removed. The presence of water bottoms in any fuel system, particularly during dormant periods of fully or partially fueled vehicle/equipment storage, may lead to microbiological growth in the fuel system. The microbiological organisms will not grow in the absence of water. This microbiological growth and its metabolic byproducts can plug filters, degrade fuel quality, and corrode those internal surfaces of fuel systems.

While it is preferable to control microbiological contamination by keeping the water out of the fuel cells, this practice is often not practical or even feasible. For those applications in which added protection against microbiological contamination is needed, the U.S. Army developed a

specification entitled "Diesel Fuel Stabilizer Additive." This specification is MIL-S-53021.(1)* The specification actually covers both a fuel biocide and a fuel stabilizer additive; however, this report is concerned only with the biocide. The biocide portion of MIL-S-53021 can serve a dual role, that of a biocide agent in dirty/water-containing cells, or as a biostat agent in cells/systems that are clean/free of water bottoms and microbiological debris. The biocide agent kills off the microorganisms and sterilizes the fuel cell/containment system. A biostat agent inhibits further growth of microorganisms in a relatively clean fuel cell, but will not necessarily sterilize the system.

Since the time that MIL-S-53021 was first written, the Army has adopted a policy of one fuel on the battlefield. This one fuel is JP-8, specified under MIL-T-83133. JP-8 is an aviation turbine kerosene fuel containing three specified additives. These additives are a corrosion inhibitor/lubricity enhancer additive, a static dissipator, and an icing inhibitor. The specified icing inhibitor is 2-(2-methoxyethoxy) ethanol; this compound also has some biocidal character to it. Additionally, when the specification was first written, only one biocide was qualified under the specification. Since that time, two additional products have been added to the qualified products list. This study was conducted to evaluate the efficacy of the individual biocides as well as the possible interactions (either synergistic or antagonistic) between these biocides and the icing inhibitor additive.

II. OBJECTIVE

The objective of this project is to evaluate the biocides currently qualified under MIL-S-53021 to determine additive efficacy (specifically, sterilization rates) and possible additive interactions when two or more different additives are present in the fuel. Of special interest is the interaction of the biocides with the additives found in JP-8 kerosene jet fuel.

* Underscored numbers in parentheses refer to the list of references at the end of this report.

III. APPROACH

The biocides were added singly and in combination to sterile kerosene (Jet-A) and Bushnell-Haas salts solution. Three separate organisms were used: a bacteria, a fungus, and a yeast. The samples were monitored periodically for presence of viable organisms and changes in fuel quality.

IV. EXPERIMENTAL

A. Test Organisms

Three microbes were used for this series of evaluations: a bacteria, *Pseudomonas aeruginosa*; a yeast, *Candida tropicalis*; and a fungus, *Cladosporium resinae*. Each of the microbes was precultured as a separate pure culture on Bushnell-Haas medium with 2-percent sterile kerosene as the carbon source. The preculture incubation period varied between 1 and 3 weeks. Biomass was harvested from each individual culture by centrifugation of the broth. The biomass pellets were resuspended in sterile Bushnell-Haas salts solution. The resuspended broths were then mixed in equal parts to form the mixed culture inocula solution for the screening tests.

B. Test Fuel

A commercially available ASTM D 1655 Jet A-1 kerosene was used for the evaluations. The fuel was clay treated as outlined in ASTM D 5001. The clay-treated fuel was sterilized by filtration through 0.2-μm pore size Acrodisc CR filters. In all cases, the additives were added to the fuel before it was subdivided into the smaller test containers.

C. Bushnell-Haas Salts Solution (2)

Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$), g	0.2
Calcium Chloride ($CaCl_2$), g	0.02
Potassium Phosphate, Monobasic (KH_2PO_4), g	1.0
Ammonium Nitrate (NH_4NO_3), g	1.0
Potassium Phosphate, Dibasic (K_2HPO_4), g	1.0
Ferric Chloride ($FeCl_3 \cdot 6\ H_2O$), Concentrated Solution 15 g/25 mL Aqueous Solution, drops	2
Distilled Water, mL	1000

Adjust to pH 6.8 to 7.0 with dilute sodium hydroxide (NaOH) before sterilization.

D. Biocides

Biocide A: Mixture of morpholine and related, substituted morpholines.

Biocide B: Mixture of substituted dioxaborinanes.

Biocide C: Same as Biocide B with an added fuel stabilizer. (Note: This additive is not qualified under MIL-S-53021. It was included in this study as a comparison since it includes the fuel stabilizer.)

Biocide D: Mixed isothiazolinones.

Biocide E: 2-(2-methoxyethoxy) ethanol (also known as diethylene glycol monomethyl ether or di-EGME). This is the fuel system icing inhibitor routinely added to JP-8.

E. Evaluation Procedures

Three separate procedures were used to evaluate the biocides. Each is described separately:

1. Procedure 1

The first set of tests was conducted to evaluate biocide efficacy and to look for any gross effects the biocides might have on the fuel. For this series of tests, sterile fuel and Bushnell-Haas solution were added to 250-mL Erlenmeyer flasks. Two different fuel/water proportions were used: 50:50 fuel/water and 95:5 fuel/water. The 50:50 proportion is prescribed in MIL-S-53021. The 95:5 proportion was used as a proportion that is closer to what might be found in the field.

At the time of test initiation, 0.5 mL of inoculum solution was added to flasks containing 50 mL of Bushnell-Haas solution. This step was followed by adding 50 mL of kerosene/additives to the flasks. For the flasks containing only 5 mL of Bushnell-Haas solution, 50 µL of inoculum solution was added to the Bushnell-Haas, followed by 95 mL of kerosene/additive. Duplicate flasks were prepared for each Bushnell-Haas/fuel/additive combination. One set (in duplicate) of control flasks consisting of Bushnell-Haas, kerosene, and inocula without biocides was also prepared. The control samples served to validate the continuing microbe viability and kerosene-degrading ability.

All flasks were incubated on an incubator shaker at 25° to 27°C and 150 rpm for at least 28 days.

The concentration of biocide used was corrected for the ratio of fuel/water used in the test. For tests using 95 mL of kerosene, the biocide concentration was simply the Recommended Effective Concentration (REC). For tests using 50 mL of kerosene, the following equation was used:

$$C_B \text{ (test)} = \text{REC}\left[\frac{P + 1}{P}\right]$$

where: C_B (test) = biocide concentration for the test

REC = manufacturer's recommended effective concentration

P = fuel/water partition coefficient.

The amount of biocide added to the fuel in each sample is listed in TABLE 1.

TABLE 1. Biocide Concentrations

Biocide	REC ppm	REC vol%	Density, g/mL	P, Fuel/Water	Volume Added, µL 95:5	Volume Added, µL 50:50
A	500	0.036	1.10	3.850	36	45
B	270	0.021	1.05	0.004	21	5270
C	270	0.021	1.05	0.004	21	5270
D	125	0.010	1.044	0.176* (CMI) 0.003 (MI)	10	67
E	--	0.15	1.01	--+	150	150

* The partition coefficient of CMI (5-Chloro-2-Methyl-4-Isothiazolin-3-one) was used for the calculation of biocide because it is the primary active ingredient in Biocide D. If the P value for MI (2-Methyl-4-Isothiazolin-3-one) were used instead, a rather large and unrealistic concentration of CMI would result.

\+ Biocide E (di-EGME) was added at 0.15 percent of the total fuel/water volume since the biocide partitions almost entirely to the water.

The volume percent REC was calculated from the ppm REC, the density of the biocide, and the density of the kerosene, which is taken as approximately 0.8 g/mL, using the following equation:

$$\text{vol\%} = \left(\frac{\text{REC}_{\text{ppm}}}{10^6 \times \rho_B} \right) \times \rho_K \times 100$$

where: ρ_B = density of biocide
ρ_K = density of kerosene.

Each flask was visually observed three times per week for evidence of microbial growth, that is:

- Biomass film at the kerosene/water interface or at the shaking ring,
- Mycelial or fungal matter at the kerosene/water interface,
- Turbidity of the aqueous phase due to suspended biomass, or
- Gradual emulsification of some or all of the kerosene phase.

The pH of the aqueous phase was measured weekly as follows:

Using a sterile pipet, a very small amount of water was withdrawn and then placed on litmus paper of appropriate range. The pH was read by comparing the color to the color guide supplied with the paper. A sterile loop of aqueous broth from each sample flask was plated after 2 and 4 weeks incubation. This process was done so as to qualitatively determine the presence of any of the inoculated organisms. *P. aeruginosa* was plated on TGE agar, *C. tropicalis* on YM agar, and *C. resinae* on PDY agar. Similarly, a loop of the mixed culture inocula was plated at 0 days to validate its viability and content.

2. **Procedure 2**

This series of tests was conducted to evaluate the sterilization rates for each of the biocides. Test flasks were prepared as described in Procedure 1, only with 2 vol% Bushnell-Haas and 98 vol% kerosene. The biocides were added to the fuel in the same concentrations listed for 95:5 (kerosene/water) in TABLE 1. Each flask was inoculated with 20 µL of the combined inocula. The control contained no biocide. A sterile loop from the fuel/water interface was plated at 0, 1, 2, 3, 4, and 7 days after the initial inoculation to check for the presence of viable organisms. Each plate was given a qualitative rating for the amount of each type of organism present. If, when compared to the control sample, an organism showed strong growth on the plate, it was given a rating of 0.33. Absence of growth of a given organism was indicated by a rating of 0. Intermediate ratings were given to indicate lesser amounts of growth on the plate.

3. **Proced . 3**

This series of tests was conducted to evaluate the potential interactions of each of the biocides with the di-EGME found in JP-5 and JP-8 kerosene jet fuels as well as in winterized diesel fuel. The conditions for Procedure 3 were the same as for Procedure 2, except for the combination of di-EGME with the other biocides. Biocide was added to the fuel to give the REC based on 100 mL of test solution. The fuel also contained 0.15 vol% of di-EGME. The control contained

neither biocide nor di-EGME. The rating system used in Procedure 2 was also used for Procedure 3.

V. RESULTS/OBSERVATIONS AND DISCUSSION

A. Procedure 1

The pH results recorded under Procedure 1 are given in Figs. 1 and 2. The results show that, in general, the pH of the aqueous phase remained fairly constant in the samples with little or no microbe growth. In those samples where organisms continued to grow throughout the test, the pH of the aqueous phase steadily decreased. Neihof also reported this reduction in pH in a mixed culture of *C. resinae* and *Candida sp.*(3) Also, according to Neihof, this reduction in the pH of the aqueous phase may have inhibited the growth of the bacteria in the cultures.

Additional observations, recorded during the project, are listed below:

- Biocide C in the 50:50 kerosene/water samples led, upon shaking, to a near complete emulsification of the kerosene in the water phase. The continuous phase was white. A small portion of the kerosene phase would separate at the top of the mixture upon cessation of shaking.

- Upon shaking, the kerosene phase of all the 50:50 kerosene/water samples tended to form dispersed droplets (sizes ranging from less than 1 mm to 5 mm). In the 95:5 samples, the kerosene remained as one continuous phase.

- Visual observations of the turbidity and presence of biomass clumps in the aqueous phase of both 95:5 and 50:50 samples corresponded directly with the plating results at 15 days of incubation.

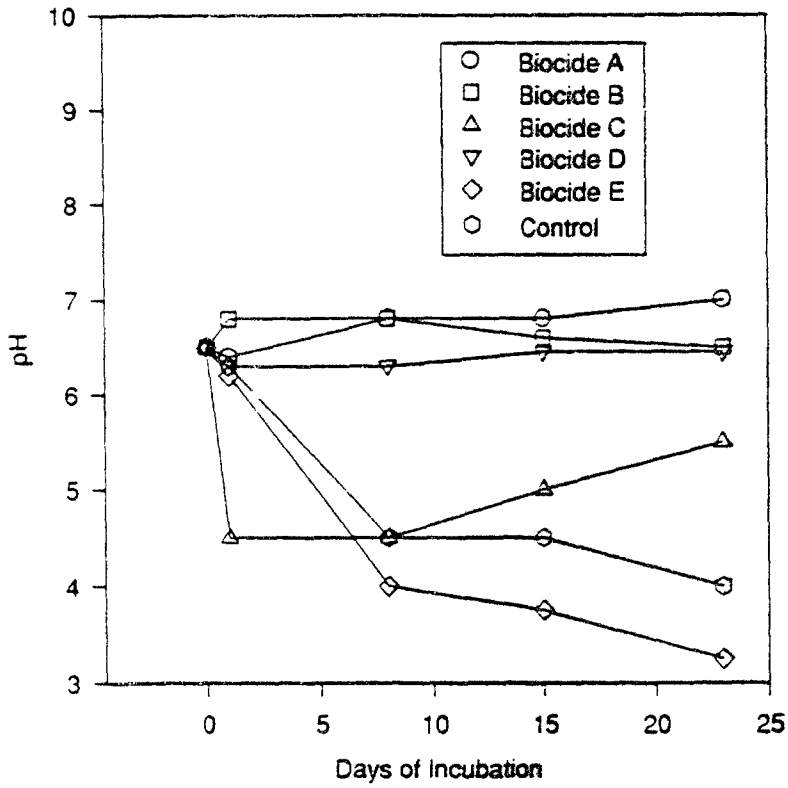


Figure 1. pH results from Procedure 1 (50 vol% kerosene:50 vol% water)

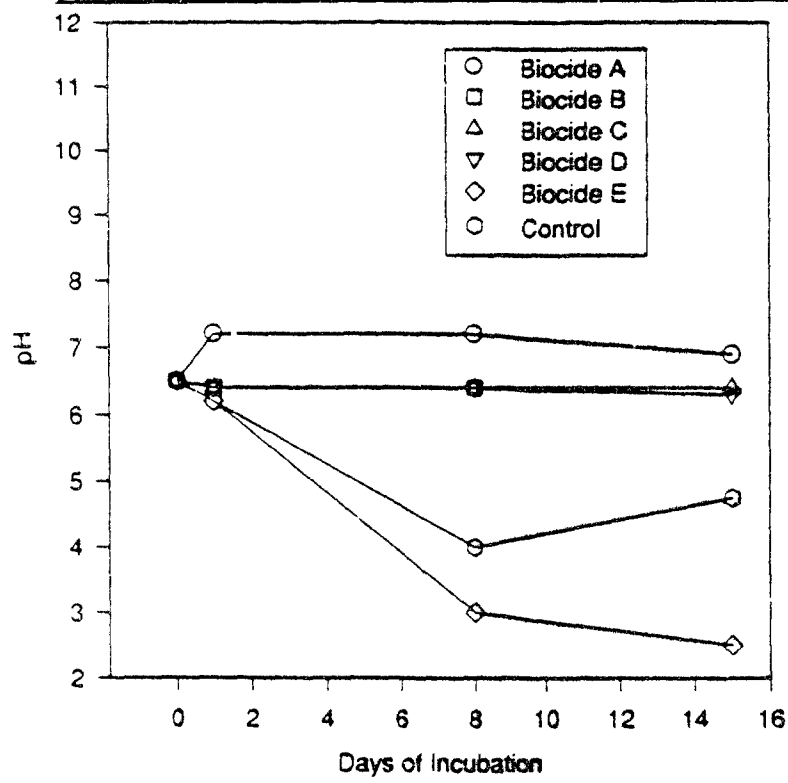


Figure 2. pH results from Procedure 1 (95 vol% kerosene:5 vol% water)

- All 95:5 samples appeared to slowly lose water, probably by evaporation, during the course of the shaking experiment. All water in these samples was gone upon observation at 23 days. This is why results are reported only up to 15 days for this set of tests.

- Biocides A, C, and D all performed appropriately in preventing microbe growth in inoculated systems, both at 95:5 and 50:50 fuel/water levels.

- Biocide B totally prevented growth in the 50:50 samples but did not prevent growth of the bacteria *Pseudomonas aeruginosa* in the 95:5 sample.

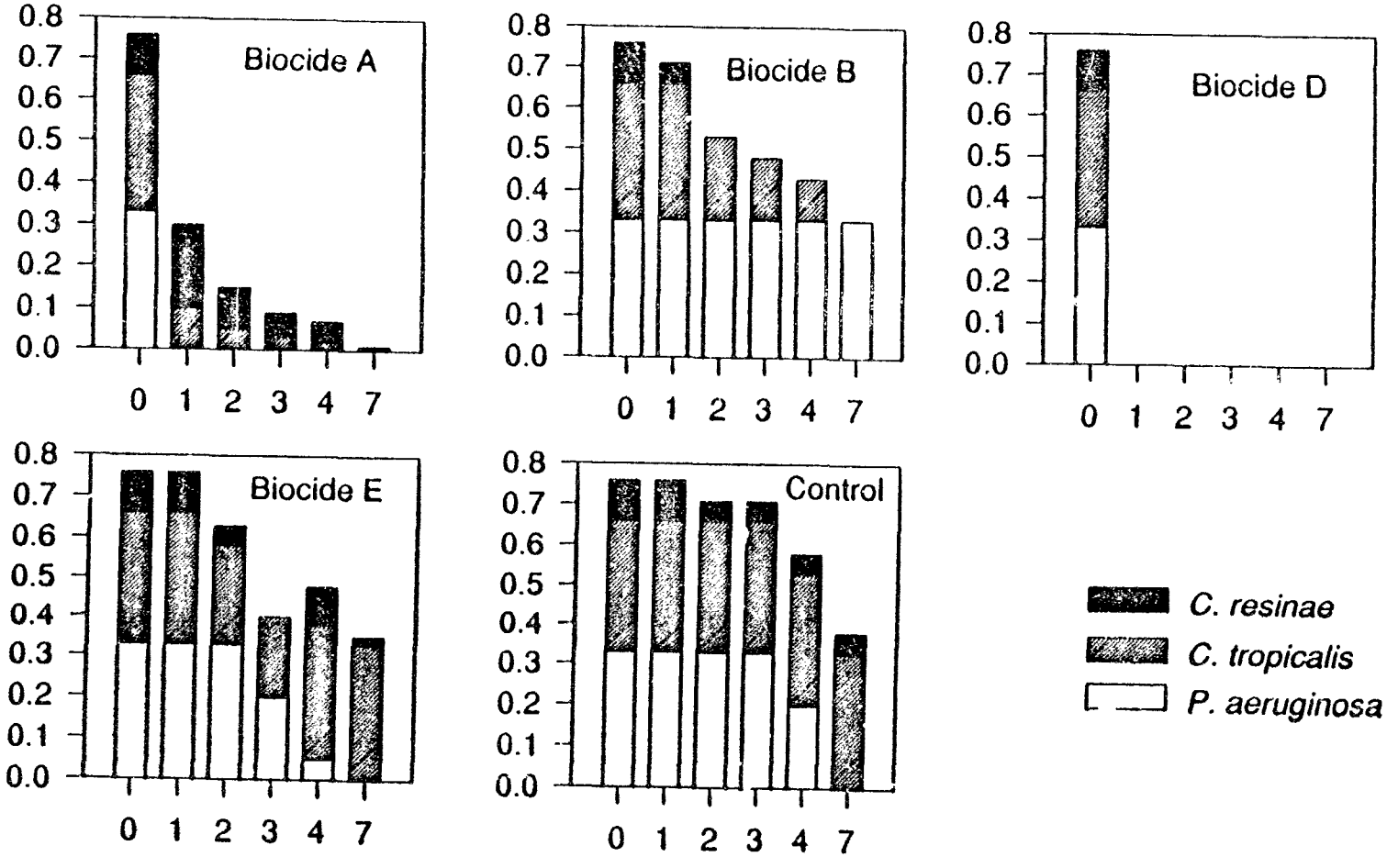
- The di-EGME possibly prevented **some** growth of the fungus *Cladosporium resinae* in both the 95:5 and 50:50 systems. The bacteria *P. aeruginosa* did not show up in either the di-EGME or control systems. This lack of *P. aeruginosa* may have been due to direct competition among the three microbes or to the pH lowering caused by extensive growth of the yeast and fungi in those systems.

B. Procedure 2

The results of this phase of the project are presented in Fig. 3. Notice that only Biocide D was successful in completely sterilizing the fuel/water system. Biocide A caused nearly complete sterilization over a period of seven days. Biocide E (di-EGME) was virtually ineffective as a biocide. If the water bottoms in the Biocide E cultures were exposed to sufficient amounts of fuel to raise the di-EGME concentration in the water to approximately 15 percent, some degree of growth inhibition would likely be noted.(3)

Additional observations are listed below:

- Biocide A caused a gradual decrease in the population of microbes present over a period of 7 days from the time of test initiation. The *Pseudomonas aeruginosa* bacteria appears



Horizontal Axis = Days Since Inoculation

Vertical Axis = Relative Microbe Presence (A rating of 0.33 indicates no reduction in growth)

Figure 3. Results from Procedure 2

to have been eliminated quickly, while the presence of both the yeast *Candida tropicalis* and the fungus *Cladosporium resinae* was decreased gradually to zero over the period observed.

- Biocide B eliminated *C. resinae* fairly quickly and *C. tropicalis* much more gradually over the 7-day test period. Biocide B did not appear to have any significant effect on *P. aeruginosa* over the entire 7-day duration of the experiment. This result is virtually the same as that obtained after 15 days in Procedure 1 for Biocide B with 95 vol% kerosene:5 vol% water, in which *P. aeruginosa* still remained at high levels on plates at 15 days into the experiment.

- Biocide D showed a complete elimination of all microbes within the first day of the biocide test. Repeated plating of the aqueous phase of the test mixture did not show any detectable recurrence of microbial growth as late as the fourth day of the test.

- Di-EGME had marginal biocidal effect. The growth of both *P. aeruginosa* and *C. resinae* were inhibited gradually over a period of 4 to 7 days. *C. tropicalis* was not substantially eliminated over the duration of the test; it remained at full strength at the 7-day time point. This result is also the same as that obtained in Procedure 1 for the di-EGME in 95 vol% kerosene:5 vol% water plated at 15 days.

- In the control sample, all three microbes remained present to some degree for most of the duration of the test. *C. resinae* was present at a reduced level, which is likely the result of an undoubtedly much lower growth rate than that of the bacteria or the yeast. *P. aeruginosa* also seems to have disappeared towards the end of the test, which could be attributed to competition and pH effects between the bacteria and the yeast. The result obtained on day 7 is virtually the same as that obtained in the plating at 15 days in Procedure 1 for either the 50:50 or 95:5 kerosene/water mixtures.

C. Procedure 3

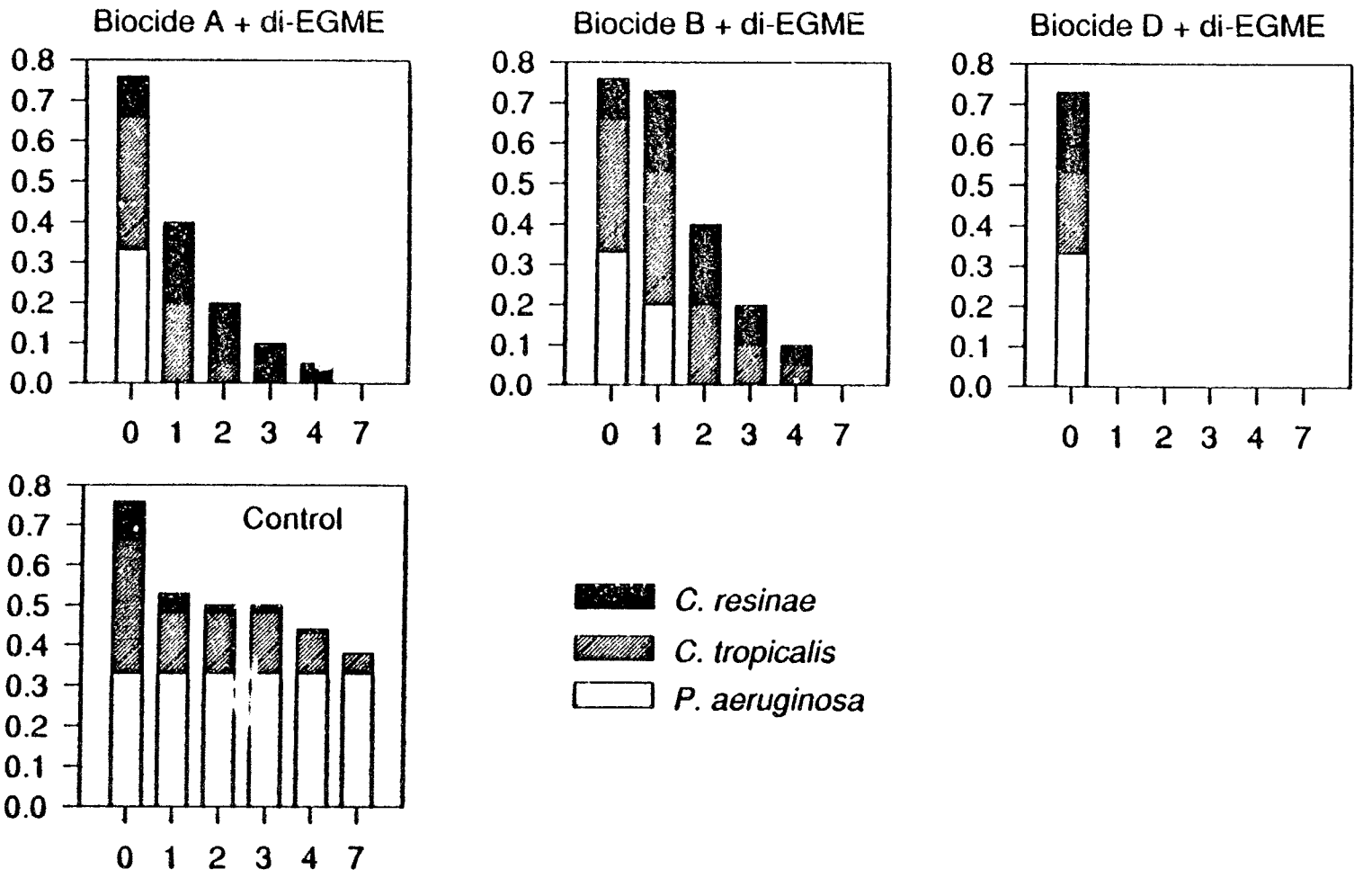
The results of Procedure 3 are presented in Fig. 4. These results are roughly equivalent to those of Procedure 2 with the exception that the presence of di-EGME in the fuel seemed to have a synergistic effect with Biocides A and B.

Additional observations are listed below:

- The Biocide A/di-EGME combination caused a gradual decrease in the population of microbes present over the 7-day test period, in a manner very similar to that observed in Procedure 2. Again, the *Pseudomonas aeruginosa* bacteria appears to have been eliminated quickly, while the presence of both the yeast *Candida tropicalis* and the fungus *Cladosporium resinae* was decreased gradually to zero over the period observed. Overall, *C. tropicalis* was the most dominant microbe in this test condition.

- The Biocide B/di-EGME combination also displayed a gradual decrease in microbe population, all the way to zero, over the 7-day test period. The *P. aeruginosa* was eliminated fairly quickly while the other two microbes, *C. tropicalis* and *C. resinae*, decreased gradually over time. In comparison with Procedures 1 and 2, it appears that a combined positive biocidal effect--Biocide B eliminating the fungus and yeast, and di-EGME eliminating the bacteria--is observed here.

- The Biocide D/di-EGME combination displayed the same biocidal effect as in the previous tests, namely, elimination of the microbial activity of all three species employed within the first 24-hour exposure period. It appears that addition of the di-EGME to Biocide D did not have any deleterious effect on the strong biocidal action of Biocide D. Repeated plating of the aqueous phase of the Biocide D test mixture did not show any detectable recurrence of microbial growth as late as the fourth day of the test.



Horizontal Axis = Days Since Inoculation

Vertical Axis = Relative Microbe Presence (A rating of 0.33 indicates no reduction in growth)

Figure 4. Results from Procedure 3

- In the control sample (no biocides or di-EGME added), a strong microbial population developed and remained present throughout the 7-day test period. The levels of both *C. tropicalis* and *C. resinae* declined with time, the former being more prominent than the latter. *Pseudomonas aeruginosa* was present at a very strong level throughout the entire test. The overall level of microbial presence observed in this test was very similar to that observed for the control in Procedure 2, though in this case, the identity of the dominant organism has changed. This change in dominancy could be the result of competition and pH effects between the bacteria and the yeast.

VI. CONCLUSIONS AND RECOMMENDATIONS

A. Conclusions

1. Concerning the Results of Procedure 1

The additional fuel stabilizer additive found in Additive C (Additive C is not approved under MIL-S-53021) appears to have the potential to emulsify water, depending on the amount of water present in the system. Some consideration of the amount of water present in the fuel may be needed when using Biocide B. The di-EGME is not an effective biocide additive for quick sterilization of a given fuel system; however, it may provide some protection with regular usage in a given fuel such as with JP-8.

2. Concerning the Results of Procedure 2

Biocide D and Biocide A eliminated virtually all active microbial contamination in these tests, though at distinctly different rates. At the kerosene/water level used, Biocide B appears to be effective primarily against the fungus and the yeast, and less so against the bacteria. Given the very low partition coefficient of Biocide B, 0.004 vol%--it almost all partitions to the water phase--it is suspected that the given REC of 0.021 vol% is insufficient for a 98:2 kerosene/water system. The partition coefficients for Biocide A and Biocide D are on the order of 1.0, and so their biocidal effect is much less sensitive to variances in the relative amount of water present

in a kerosene/water system. Such results suggest that perhaps the administration of Biocide B as a biocide should take into account the different water levels that may be present in various fuel storage and delivery systems to which it is added instead of using a single REC of 0.021 vol% across the board.

3. Concerning the Results of Procedure 3

In a manner very similar to Procedure 2, Biocide D and Biocide A eliminated virtually all active microbial contamination in these tests, though at distinctly different rates. The addition of di-EGME to these two biocides did not appear to have any discernible negative or positive effect on elimination of microbial activity. The Biocide B/di-EGME combination did show significant difference, specifically the elimination of the bacteria *P. aeruginosa*, as compared to Biocide B alone from Procedure 2.

B. Recommendations

If quick sterilization (i.e., 24 to 48 hours) of a given fuel system is required, it is recommended that either Biocide A or Biocide D be used. For routine preservation of a fuel system prior to long-term storage, any of the qualified additives would be sufficient.

It is also recommended that a study be conducted to evaluate the potential interactions of two or more of the approved biocides being mixed together. The mixing is not thought to be a potentially common problem, but it is a possibility.

VII. LIST OF REFERENCES

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3. Neihof, R.A. "Microbes in Fuel: An Overview with a Naval Perspective," *Distillate Fuel Contamination, Storage, and Handling, ASTM STP 1005*, H.L. Chesneau and M.M. Dorris, eds., American Society for Testing and Materials, Philadelphia, pp. 6–14, 1988.

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